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Zebrafish *grainyhead-like1* is a common marker of different non-keratinocyte epidermal cell lineages, which segregate from each other in a Foxi3-dependent manner

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ABSTRACT Grainyhead/CP2 transcription factor family members are widely conserved among the animal kingdom and have been implicated in different developmental processes. Thus far, nothing has been known about their roles in zebrafish. Here we identify seven zebrafish *grainyhead-like (grhl) / cp2* genes, with focus on *grhl1*, which is expressed in the periderm and in epidermal ionocyte progenitors, but downregulated when ionocytes differentiate. In addition, expression was detected in other "non-keratinocyte" cell types of the epidermis, such as *pvalb8*-expressing cells, which according to our lineage tracing experiments are derived from the same pool of progenitor cells like keratinocytes and ionocytes. Antisense morpholino oligonucleotide-based loss-of-function analysis revealed that *grhl1* is dispensable for the development and function of all investigated epidermal cell types, but required as a negative regulator of its own transcription during ionocyte differentiation. Knockdown of the transcription factor Foxi3a, which is expressed in a subset of the *grhl1* population, caused a loss of ionocytes and a corresponding increase in the number of *pvalb8*-expressing cells, while leaving the number of *grhl1*-positive cells unaltered. We propose that *grhl1* is a novel common marker of all or most "non-keratinocyte" epidermal progenitors, and that the sub-functionalisation of these cells is regulated by differential positive and negative effects of Foxi3 factors.

KEY WORDS: *grainyhead, skin, ionocyte, foxi3, p63, pvalb8, zebrafish*

Introduction

During embryonic and larval development, the zebrafish epidermis consists of two cell layers, the basal layer, which originates from the ventral/non-neural ectoderm of the gastrula embryo, and the superficial layer, the periderm, which is derived from the embryonic enveloping layer (EVL), a lineage that segregates from the deep cells (including the epidermal ectoderm) during pre-gastrula stages (Kimmel *et al.*, 1990; Le Guellec *et al.*, 2004; Sonawane *et al.*, 2005). Both layers predominantly consist of keratin-synthesising epithelial cells. In addition to these keratinocytes, the epidermis of teleosts contains a range of different cell types, such as ionocytes, which perform active ion transport and are involved in osmoregulation, mucus-secreting goblet cells, and other secretory cell types such as sacciform cells and club cells (for review, see Whitear, 1986). In zebrafish embryos and larvae, epidermal ionocytes are distributed in a punctate pattern throughout the epidermis of the trunk and tail. They are comprised of at least two types, Na^+,K^+-ATPase-rich (NaR) cells and H^+-ATPase-rich (HR) cells, which are characterised by the expression of the specific ATPase genes *atp1b1b* and

Abbreviations used in this paper: BrdU, bromodeoxyuridine; dpf, days post fertilization; EVL, enveloping layer; GFP, green fluorescent protein; grhl, grainyhead-like; hpf, hours post fertilization; HR, H^+-ATPase-rich; MO, morpholino oligonucleotide; NaR, Na^+,K^+-ATPase-rich; NCC, Na^+/Cl^--cotransporter expressing; pNB, postembryonic neuroblasts; RT-PCR, reverse transcription polymerase chain reaction.

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atp6v1al, respectively (Lin *et al.*, 2006; reviewed in Hwang and Lee, 2007). In addition, epithelial mucous cells have been described (Hsiao *et al.*, 2007; Shih *et al.*, 2007). The cell bodies of these different "non-keratinocyte" cells are located within the basal layer of the epidermis, with characteristic protrusions that extend through the outer periderm towards the surface of the fish (Whitear, 1986).

Cell lineage tracing experiments have shown that basal keratinocytes and ionocytes are derived from a common pool of precursor cells from the ventral ectoderm (Jänicke *et al.*, 2007). Foxi3 transcription factors are necessary for ionocyte specification, while Notch ligands specifically made in developing ionocytes prevent a further expansion of the ionocyte lineage by signalling to neighbouring epidermal cells, thereby preventing *foxi3* expression in these cells and allowing them to adopt a keratinocyte fate (lateral inhibition) (Jänicke *et al.*, 2007; Hsiao *et al.*, 2007). Within the keratinocyte lineage, the transcription factor DNp63 is crucial for proliferation, although most likely dispensable for differentiation of these cells (Bakkers *et al.*, 2002; Lee and Kimelman, 2002). However, the exact mechanisms controlling the initial segregation steps of the different epidermal cell lineages are still poorly understood.

Members of the Grainyhead/CP2 transcription factor family are highly conserved across the animal kingdom and are involved in a variety of developmental processes. The family is split into two divisions, the Grainyhead and the CP2 subgroup. The first Grainyhead protein was identified in *Drosophila melanogaster*, based on its binding to neuronal cis elements, and initially called NTF-1 or Elf-1, but renamed Grainyhead according to its mutant (Dynlacht *et al.*, 1989; Bray *et al.*, 1989; Bray and Kafatos, 1991), while CP2 was first discovered in mouse, based on its binding to the *globin* promoter (Lim *et al.*, 1992).

The fruitfly has one member of each subgroup, *D-grainyhead* and *D-Cp2.* In mammals, three *grainyhead*-like genes have been described, *Grainyhead-like1* (also known as *Mother-of-grainyhead* or *Lbp-32*), *Grainyhead-like2* (also known as *Brother-of-mammalian-grainyhead*, or *Cp2-like3*) and *grainyhead-like3* (also known as *Sister-of-mammalian-grainyhead*, *Cp2-like4* or *Get-1*). The CP2 subgroup also consists of three genes in mammals, *Cp2, Cp2-like1* and *Lbp-1a* (Venkatesan *et al.*, 2003; Wilanowski *et al.*, 2002).

In *Drosophila,* tissue-specific splice variants of *grainyhead* are required for epidermal specification (Bray and Kafatos, 1991) as well as neuroblast differentiation (Maurange *et al.*, 2008; Almeida and Bray, 2005; Cenci and Gould, 2005). Mammalian *Grainyhead-like2* is required for maturation of fluid/solute transporting epithelial ducts in both the salivary gland and the kidney in mice (Yamaguchi *et al.*, 2006) and has also been linked to age-related deafness in humans (Van Laer *et al.*, 2008). Mice deficient in *Grainyhead-like3* show abnormalities in the granular and cornified layer of the skin and in cutaneous wound healing, indicating that the gene is required for proper terminal differentiation of the epidermis and for epithelial morphogenesis (Ting *et al.*, 2005; Hislop *et al.*, 2006; Yu *et al.*, 2006; Yu *et al.*, 2008). A similar differentiation-promoting role has been demonstrated for *Xenopus Grhl2*, which upon forced expression induces genes specific for differentiated superficial cells in the deep proliferating epidermal cells (Chalmers *et al.*, 2006). In addition, *Xenopus Grhl1* disruption leads to defects in epidermal differentiation, as indicated by the loss of keratin genes expression (Tao *et al.*, 2005), while mouse *Grhl1* mutants display skin and hair defects partly caused by reduced expression of the genes encoding desmosomal cadherins (Wilanowski *et al.*, 2008).

Here we report the identification of seven members of the Grainyhead/CP2 family in the zebrafish genome. The genes cluster into the six already described subgroups, with *grhl2* as the only duplicated pair. The aim of this work was the analysis of *grainyhead-like1* (*grhl1*), which is expressed in the periderm (outer layer of the embryonic skin) until 2 days post fertilisation (dpf), and in single cells spread throughout the epidermis of the developing embryo. Performing fluorescent double *in situ* hybridisations, we show that this punctate pattern reflects transient *grhl1* expression in ionocyte progenitors, while expression is downregulated when ionocytes start to differentiate. In addition, *grhl1* expression was detected in other non-keratinocyte skin cell types deriving from the same pool of epidermal progenitors, such as *pvalb8*-expressing putative mucous cells. We propose that *grhl1* marks the progenitor stages of various cell lineages in the skin of the developing zebrafish larvae. Furthermore, we show that loss of *foxi3*a, which has a negative effect on ionocyte markers such as *atp1b1b* and *atp6v1al*, has a positive effect on *pvalb8* expression, suggesting that Foxi3 transcription factors induce ionocyte differentiation in epidermal cells that would otherwise become *pvalb8*-expressing cells.

Results

grainyhead-like1 (grhl1) *is expressed in skin cells of the zebrafish larvae*

Performing *in situ* hybridisations we found that during the first 3 days of development, *grhl1* was expressed in a punctate pattern throughout the basal epidermis of the zebrafish larvae. During cleavage (Fig. 1A), blastula and gastrula stages (data not shown), no *grhl1* expression was detectable, indicating the lack of maternal mRNA contribution. First specific expression was observed around the 2-somites stage in distinct cells spread across the yolk of the embryo (Fig. 1B), resembling the previously described pattern of the *foxi3* genes at the same stage (Solomon *et al.*, 2003; Hsiao *et al.*, 2007; Jänicke *et al.*, 2007). As somitogenesis progresses, *grhl1*-positive cells also appeared in the basal epidermis over the developing yolk sac extension and trunk (Fig. 1 C,D) as well as in the olfactory placodes (Fig. 1C, arrowhead). In addition, very faint expression was detected throughout the entire skin of the larvae until 2 dpf. This staining was restricted to cells of the superficial layer (also called enveloping layer (EVL) or periderm), which are characterised by their hexagonal cell shape (Fig. 1 E-E´´, black arrows; compare with single basal cell showing strong *grhl1* expression indicated with red arrow). At 3 dpf, the punctate expression of *grhl1* over the trunk began to vanish, while expression started in the branchial arch regions (Fig. 1 F and G). At 5 dpf, *grhl1* was strongly expressed in the branchial arches as well as in the nasal pits (Fig. 1 H and I). At all stages investigated, no signal was detected with a *grhl1* sense probe (Fig. 1E´´ and data not shown).

Seven members of the Grainyhead/Cp2-family can be identified within the zebrafish genome

To identify other zebrafish *grhl* genes, zebrafish genome

databases were searched using BlastN and TBlastN with *Drosophila grh* and *Drosophila cp2* as queries. Seven genes located on five different chromosomes were identified (Fig. 1J). When a phylogenetic tree was constructed, including previously published sequences of Grhl/Cp2 proteins from human, mouse, chicken and *Xenopus*, the identified zebrafish genes clustered into the six already described subgroups of the vertebrate Grainyhead/CP2 family (Fig. 1J, Wilanowski *et al.*, 2002; Venkatesan *et al.*, 2003). For *grhl1*, which is located on chromosome 17, no paralogue was identified. In addition, only one zebrafish orthologue of each of mammalian *Grainyhead-like3, Cp2, Cp2-like1* and *Lbp-1a* was identified on chromosome 17 (z-*grhl3*), chromosome 23 (*z-cp2*), chromosome 9 (*z-cp2-like1*) and chromosome 19 (*z-lbp-1a*), respectively. However, two sequences, placed on chromosome 16 and 19, clustered with Grainyhead-like 2 proteins. These genes were therefore called *z-grhl2a* and *z-grhl2b,* respectively.

Transcripts of all genes except *z-lbp-1a* could be detected by RT-PCR during the first three days of development (data not shown), but of the five other *grh/cp2* genes that were positive in the RT-PCR, only expression of *grhl2a, grhl2b* and *grhl3* was high enough for detection in our whole mount *in situ* hybridisations (Fig. 1 K-T). This difference in sensitivity of both methods has also been described by others (Kochilas *et al.*, 2003; Zhao *et al.*, 2005; Xu *et al.*, 2006). While *grhl2a* expression was restricted to the olfactory and otic placodes as well as to the pronephros from mid somitogenesis until 1 dpf (Fig. 1 K-L), *grhl2b* was ubiquitously expressed during gastrulation and the first day of development (Fig. 1 M-O´). *grhl3* displayed expression in the periderm of the skin, which started at late gastrula stages (Fig. 1 P-Q') and persisted, although at lower levels, during segmentation (Fig. 1 S,T), when the periderm showed strong *grhl1* expression. In addition, but unlike *grhl1*, *grhl3* was expressed in pharyngeal tissue of segmentation embryos (Fig. 1 R, arrow). In reverse, *grhl3* lacked the punctate expression in the basal epidermis displayed by *grhl1* (Fig. 1 S,T; data not shown). In the Zebrafish Model Organism Database ZFIN, the same expression pattern has been published for *grhl3* under the name si:dkey-221l4.7 (Thisse *et al.*, 2001; http://zfin.org).

grhl1 *is expressed in ionocyte precursor cells but switched off in differentiated ionocytes*

The expression of *grhl1* in single cells throughout the skin during the first three days of development, followed by appearance in the branchial arch regions, resembles the expression pattern described for markers of differentiating HR- and NaR-type ionocytes (Varsamos *et al.*, 2005; Hsiao *et al.*, 2007; Jänicke *et al.*, 2007).

We have previously shown that ionocytes are derived from the

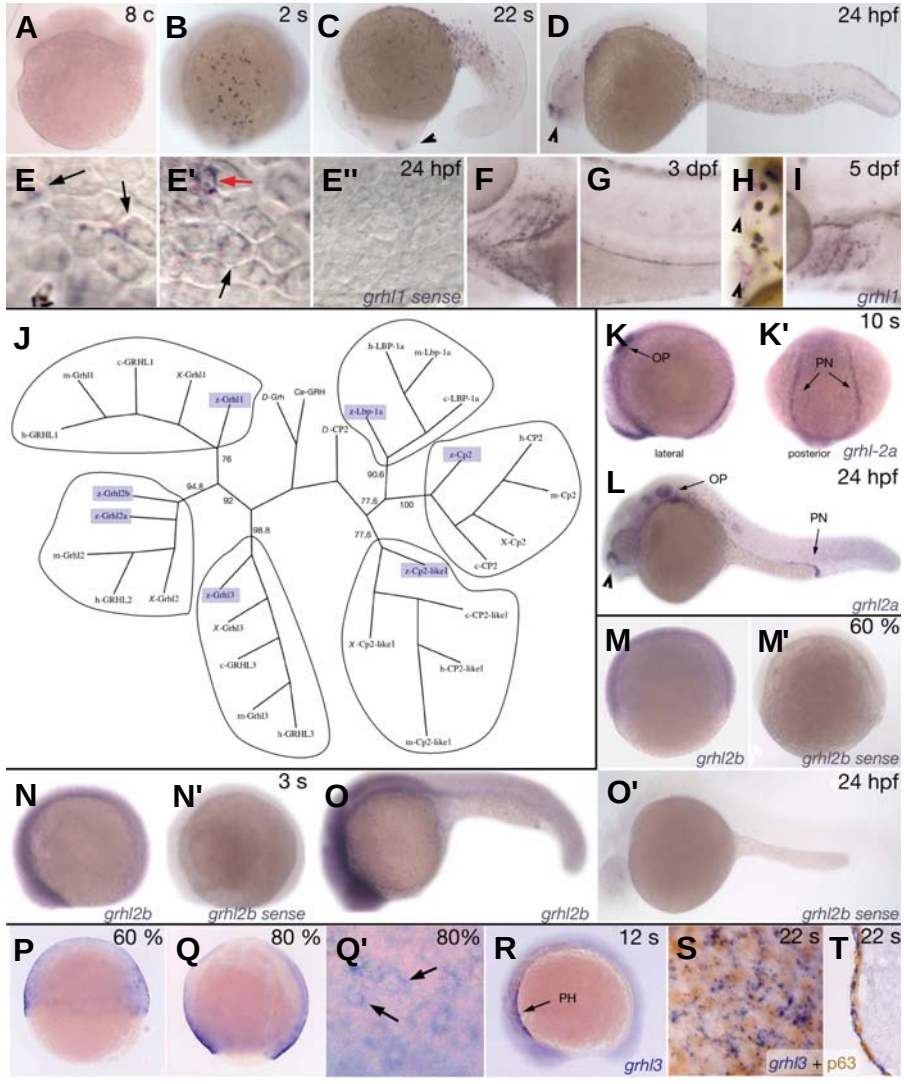


Fig. 1. Of all zebrafish *grhl-cp2* homologs, only *grhl1* and *grhl3* display specific expression in the embryonic skin. *All panels except (J) show whole mount* in situ *hybridisations of wild-type embryos at stages indicated in the upper right corner and probes indicated in the lower right corner (c, cells; s, somites; %, % epiboly).* **(A-I)** *Expression of* grhl1; **(E´´)** *control staining with sense probe. Black arrowheads in (C,D,H,L) point to the olfactory placodes.* **(E-E´´)** *High magnifications of the tail fin, with peridermal cells in focus (recognisable by their hexagonal shape). Black arrows point to staining in the superficial peridermal cells, the red arrow in (E') to a single cell in the underlying basal epidermis that expresses high levels of* grhl1. **(K-L)** *Staining for* grhl2a. **(M-O´)** *Expression of* grhl2b *with an antisense (M-O) and sense probe (M´-O´).* **(P-T)** *Expression of* grhl3*, with (Q') displaying a magnified view on the enveloping layer (future periderm; black arrows point to single cells) of the embryo shown in (Q). All panels except (F,H,K´ ,T) show lateral views with anterior/vegetal to the left.* **(F,H)** *Ventral views with anterior to the left; (K´) is a posterior view with dorsal up, and (T) shows a transverse section at the trunk level.* **(J)** *An unrooted phylogenetic tree based on positional variation within regions of sequences that aligned across all identified (boxed) and published Grainyhead/CP2 proteins using the neighbor-joining algorithm. Bootstrap values in % based on 1000 replicates are indicated for the major branching points. Abbreviations: OP, otic placode; PH, pharynx; PN, pronephros.*

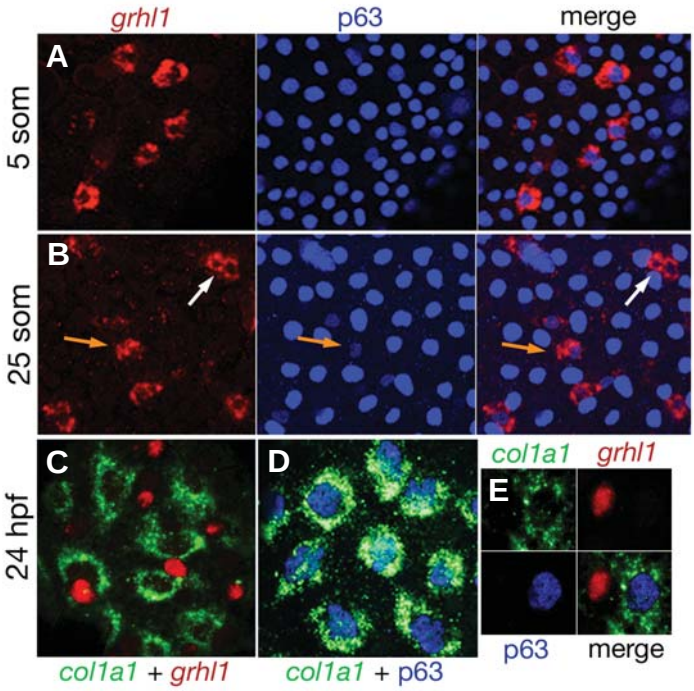


Fig. 2 (Above). *grhl1* is transiently expressed in a subset of epidermal precursors, but absent from differentiated basal keratinocytes. (A,B) *Fluorescent* in situ *hybridisation for* grhl1 *(in red) combined with immunostaining for p63 (in blue).* **(C)** *Double fluorescent* in situ *hybridization for* col1a1 *(in green) and* grhl1 *(in red).* **(D)** *Fluorescent* in situ *hybridisation for* col1a1 *(in green) combined with immunostaining for p63 (in blue).* **(E)** *A triple staining for* col1a1 *mRNA,* grhl1 *mRNA and p63 protein (three single channels plus merged image). For panels (C-E), embryos injected with* grhl1 *splice MO were used, leading to accumulation of* grhl1 *mRNA in the nucleus and facilitating the analysis of the obtained patterns (compare with Figs. 6 and S7).*

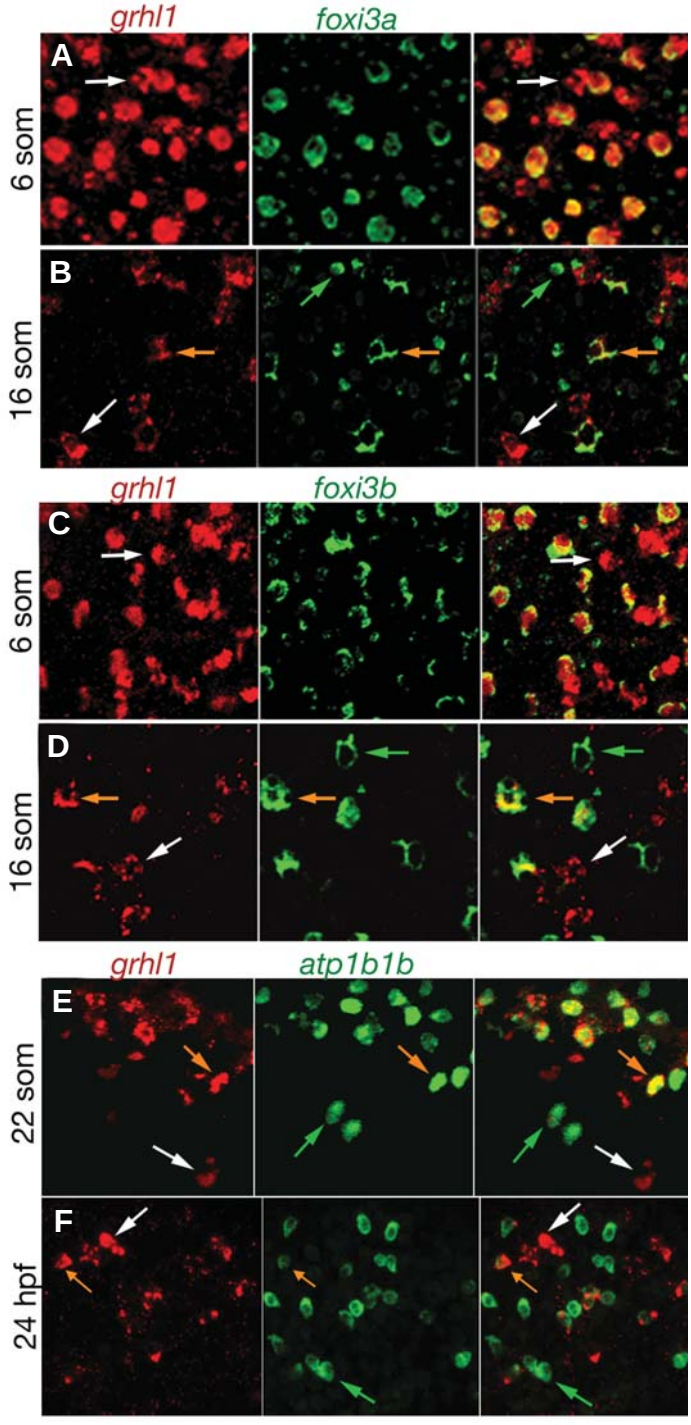


Fig. 3 (Right). *grhl1* is transiently expressed in ionocytes. *All panels show double fluorescent* in situ *hybridisations with probes indicated above the panels. The right picture of each panel shows the merge of the two left pictures. Stages are indicated at the left. All panels show cells of wild-type embryos over the yolk sac (5 -16 som) or over the anterior half of the trunk (22 som - 24 hpf). White arrows mark cells expressing only* grhl1*, green arrows mark cells only expressing* foxi3a **(B)**, foxi3b **(D)**, *or* atp1b1b **(F)**, *and orange arrows mark cells co-expressing the two indicated genes.*

same precursor cells as keratinocytes within the ventral ectoderm of the zebrafish gastrula (Jänicke *et al.*, 2007). The transcription factor DNp63, a crucial regulator of keratinocyte proliferation, (Bakkers *et al.*, 2002; Lee and Kimelman, 2002), is temporarily expressed in all epidermal progenitor cells of the non-neural ectoderm, while later, expression is only maintained in basal keratinocytes (Fig. 2D). Consistently, it is temporarily made in ionocyte progenitors, but lost after the onset of expression of *foxi3a* and *foxi3b*, essential regulators of NaR and HR ionocyte specification (Hsiao *et al.*, 2007; Jänicke *et al.*, 2007).

Fluorescent *in situ* hybridisations for *grhl1* in combination with anti-p63 immunostainings revealed that at early somite stages, *grhl1* positive cells contained normal or only moderately reduced levels of nuclear DNp63 (Fig. 2A). At late somitogenesis, however, DNp63 had disappeared (Fig. 2B, white arrow) from *grhl1*-positive cells, or was present at much lower levels than in the *grhl1*-negative presumptive keratinocytes (Fig. 2B, orange arrow). Consistently, and in contrast to p63, *grhl1* expression was absent from differentiated basal keratinocytes at 24 hpf (30-somite stage), as revealed by double fluorescent *in situ* hybridizations for *grhl1* and the keratinocyte markers *collagen 1a1* (*col1a1*; Fig. 2 C,E). Together, this indicates that *grhl1* labels non-keratinocyte progenitors of the non-neural ectoderm.

To further investigate the identity of *grhl1* expressing cells, we analysed its expression in comparison to that of *ATPase* genes,

markers for differentiated ionocytes, and of *foxi3* genes, which mark differentiated ionocytes as well as their progenitors. At the 6-somite stage all *foxi3a* and *foxi3b* cells expressed *grhl1* (Fig. 3 A,C), while some *grhl1*-positive, *foxi3*-negative cells could also be detected (Fig. 3 A,C, white arrow). Such *grhl1* single positive cells were also observed at the 16-somite stage (Fig. 3 B,D, white arrow). They could either represent less advanced ionocyte precursors prior to the onset of *foxi3* expression, or another, *foxi3*-independent non-keratinocyte epidermal cell type. In contrast to the 6-somite stage, however, 16-somite stage embryos also contained *foxi3*-positive, but *grhl1*-negative cells (Fig. 3 B,D, green arrows) in addition to *foxi3*, *grhl1* double positive (Fig. 3 B,D, orange arrows) and *grhl1* single positive cells (white arrows). These *foxi3* single positive cells might represent more advanced, differentiating ionocytes, suggesting that in contrast to *foxi3* genes, *grhl1* is switched off during ionocyte differentiation. Corresponding temporal shifts in co-expression were observed for *grhl1* and *ATPase* genes. At the 22-somite stage (20 hpf), approximately 36% (34.2±2.18 / 93.3±5.14, n = 5 sections) of NaR cells labelled by *atp1b1b* mRNA were also positive for *grhl1* (Fig. 3E, orange arrow). In contrast, at the 30-somite stage (24 hpf) only approximately 18 % of NaR cells (19.5±3.25 / 109.2±3.76, n=10) and 8 % of HR cells (5.0±1.14 / 62±2.86; n=10), characterised by *atp6v1al*, expressed *grhl1* (Figs. 3 F and S7C, orange arrows; Fig. 6N), while the number of NaR cells lacking *grhl1* expression had increased accordingly (Fig. 3 E,F, green arrows; Fig. 6N). At both 20 and 24 hpf, however, embryos contained comparable numbers of *grhl1*-positive, *ATPase*-negative cells, representing either ionocyte precursors and/or another non-epidermal cell type (Fig. 3 E,F, white arrows; Fig. 6N) (20 hpf: ~63%, 56.3±3.35 / 89.3±4.24, n=5; 24 hpf: ~54%, 31.0±3.41 / 57.2±3.50, n=10).

grhl1 *is expressed in* pvalb8*-positive cells, which are distinct from NaR and HR ionocytes, but which also derive from the common pool of epidermal progenitors*

To further analyse the nature of the *grhl1*-positive, but *foxi3*- and *ATPase*-negative cells, we studied the expression of *pvalb8* (initially termed *pvalb3a*, but renamed according to http://zfin.org), which according to Hsiao *et al.* labels epidermal mucous cells (Hsiao *et al.*, 2002). Indeed, in our fluorescent double *in situ* hybridisations, *pvalb8*, *atp1b1b* and *atp6v1al* displayed complementary punctate expression patterns in 24 hpf wild-type embryos (Fig. 4A ,B). Carrying out *grhl1, pvalb8* double labellings, we further found that at 24 hpf, approximately 3 % (1.7±0.4/57.2±3.5, n=10) of the *grhl1*-positive cells also displayed expression of *pvalb8* (Figs. 4C and 6N), suggesting that similar to ionocytes, *grhl1* is transiently expressed in the progenitors of *pvalb8* cells.

We have previously shown that NaR and HR ionocytes derive from the same pool of epidermal progenitors as the keratinocytes (Jänicke *et al.*, 2007). To test whether this is also true for *pvalb8*-expressing cells, the other *grhl1*-positive subpopulation of non-keratinocyte cell types, we carried out similar cell lineage analyses, transplanting single GFP-labeled cells of the ventral (epidermal) ectoderm of an early gastrula embryo (6 hpf) homotopically into an unlabelled host of the same age, and staining the derivatives of the transplanted cell at 24 hpf for p63 protein (keratinocyte

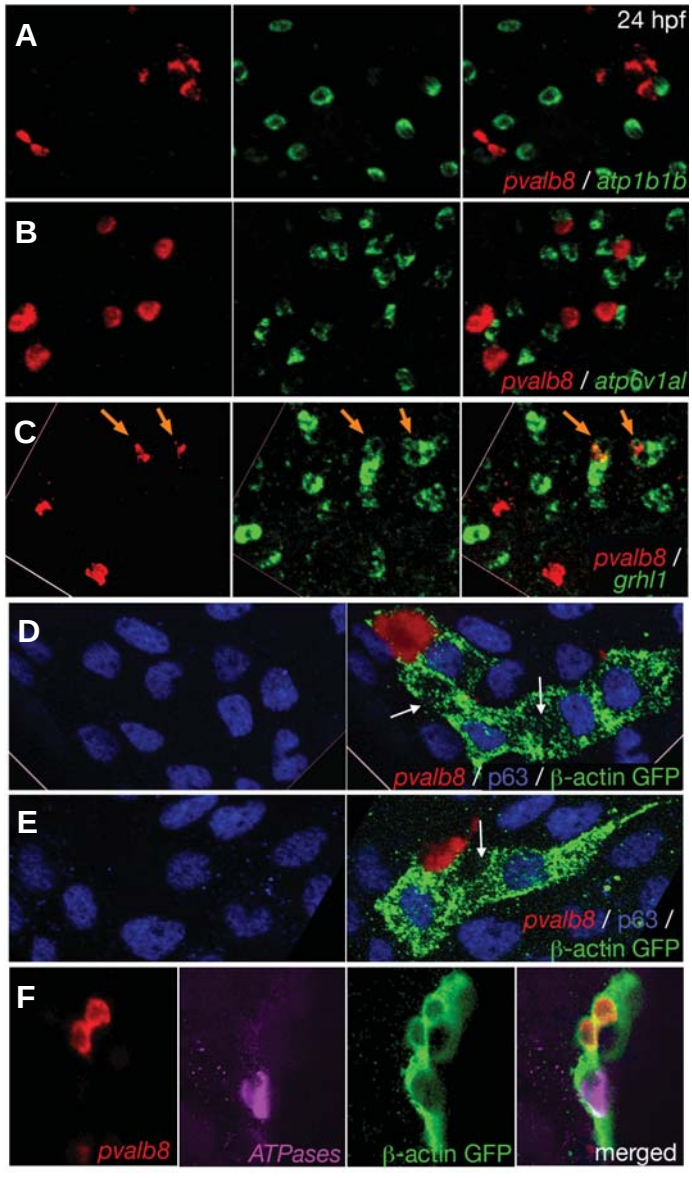


Fig. 4. *pvalb8*-expressing cells are distinct from NaR and HR ionocytes, but also display transient *grhl1* expression and derive from the common pool of epidermal progenitors. *All panels show* in situ *hybridisations and/or immunostainings with the probes and antibodies indicated in lower right corners.* **(A,B)** *Double fluorescent* in situ *hybridisations of wild-type embryos at 24 hpf, revealing that* pvalb8*-positive cells lack expression of* atp1b1b *(A) and* atp6v1al *(B).* **(C)** *Double fluorescent* in situ *hybridisations of wild-type embryos at 24 hpf, revealing co-expression of* grhl1 *and* pvalb8 *in cells indicated by orange arrows. The left and middle pictures of each panel show the single channels of the merged image shown in the right picture.* **(D-F)** *Clones of epidermal cells of wild-type embryos at 24 hpf, after homotopic and homochronic transplantation of single GFP-transgenic cells into the ventral ectoderm at 6 hpf (shield stage). The left pictures of* **(D,E)** *show an anti-p63 immunostaining of keratinocytes (in blue), the right pictures show an overlay of the p63 immunostaining with an* in situ *hybridisation for* pvalb8 *transcripts (in red) and an anti-GFP immunostaining for the descendants of the transplanted cell (in green). The white arrows point to p63,* pvalb8*-double negative cells in the clone, which might represent an NaR or HR ionocyte.* **(F)** *From left to right:* in situ *hybridization for* pvalb8 *transcripts (in red),* in situ *hybridization for* atp1b1b *and* atp6v1al *transcripts (in pink), anti-GFP immunostaining for the descendants of the transplanted cell (in green), merged image.*

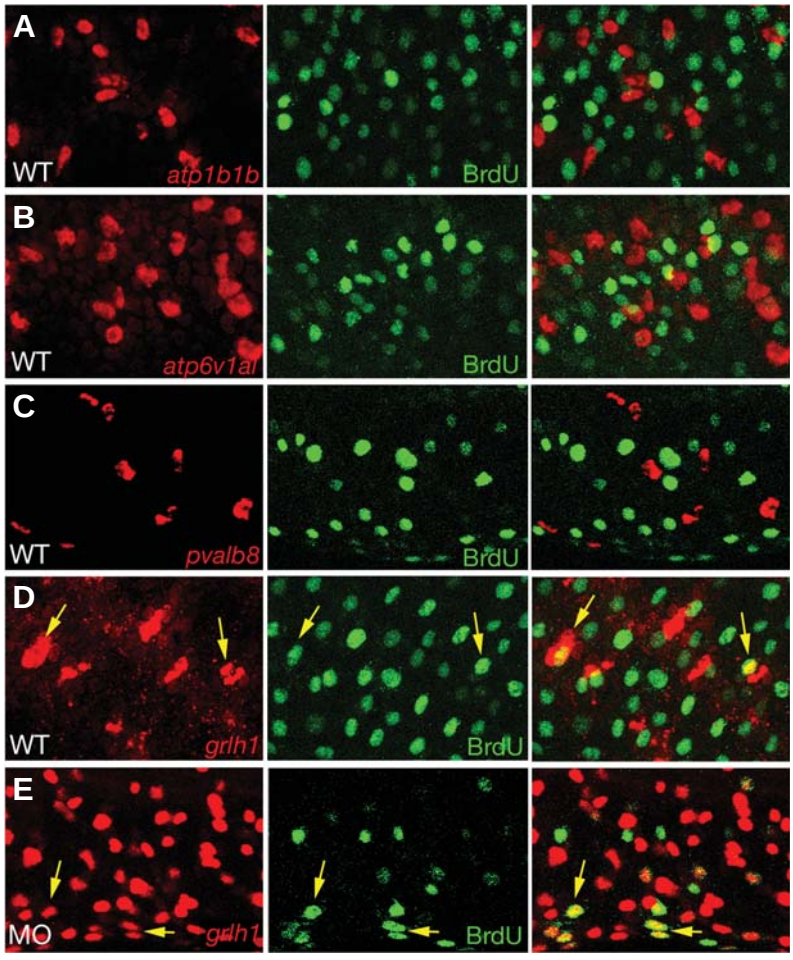


Fig. 5. *grhl1* is expressed in proliferating cells. *All panels show confocal sections of embryos at 24 hpf that had been incubated with BrdU from 16 – 24 hpf, and after fluorescent* in situ *hybridization with probes indicated in red (left panels), and after anti-BrdU immunostaining (middle panels); right panels show merged images.* **(A-D)** *Wild-type embryos;* **(E)** grhl1 *morphant injected with splice-MO (compare with Fig. 6 and Supplementary Fig. S7). Yellow arrows in* **(D,E)** *point to* grhl1*, BrdU-double positive cells. Differentiated ionocytes and pvalb8-positive cells lack BrdU incorporation* **(A-C)***, whereas over one quarter of* grhl1*-positive cells have undergone DNA replication between 16 and 24 hpf* **(D)** *(<28%, 17.6±2.83 / 62±4.45, n = 10 sections).* **(E)** *In morphant embryos, the absolute number of* grhl1*-positive cells increases enormously (see also Fig. 6N), whereas the absolute number of* grhl1*, BrdU-positive cells remains similar (19.3±2.44 / 341±8.91, <5.5%, n = 10 sections), suggesting that the extra* grhl1 *cells represent differentiated non-keratinocyte epidermal cells, rather than their precursors.*

marker) and *pvalb8* mRNA. Clusters of four to eight cells were detected that consisted of one *pvalb8* cell, several keratinocytes and one to two p63, *pvalb8* double-negative cells (Fig. 4 D,E; n=5/5 clones). Double *in situ* hybridization further revealed the co-existence of *pvalb8*- and *atp1b1b*/*atp6v1al*-positive cells in such GFP-positive clusters (Fig. 4F; 3 clones). These finding show that epidermal *pvalb8* cells derive from the ventral ectoderm and share the same progenitors as keratinocytes and ionocytes.

In contrast to differentiated ionocytes and pvalb8*-positive cells,* grhl1*-positive cells undergo cell proliferation*

To further look into the notion that *grhl1* is expressed in precursors of, rather than fully differentiated non-keratinocyte skin cells, we carried out BrdU incorporation studies to label cells undergoing DNA replication, in combination with *in situ* hybridization for *grhl1*, *pvalb8* or *ATPase* transcripts. Interestingly, we found that *grhl1*-positive cells do not only incorporate BrdU during early segmentation stages (10 hpf – 16 hpf; data not shown), when the first ionocytes are formed, but also during mid-segmentation stages (15 hpf – 24 hpf; Fig. 5D). In contrast, *ATPase*- or *pvalb8*-positive cells of sibling embryos treated during the same mid-segmentation interval were all BrdU-negative (Fig. 5 A-C). Together with the aforementioned lineage tracing and co-expression analyses, these findings indicate that during the first day of development *grhl1* is temporarily expressed in proliferating progenitors of a number of different, non-keratinocyte skin cells that derive from the same pool of epidermal precursor cells, while expression is down-regulated during cell differentiation.

Grhl1 is dispensable for proper specification and function of the different skin cells

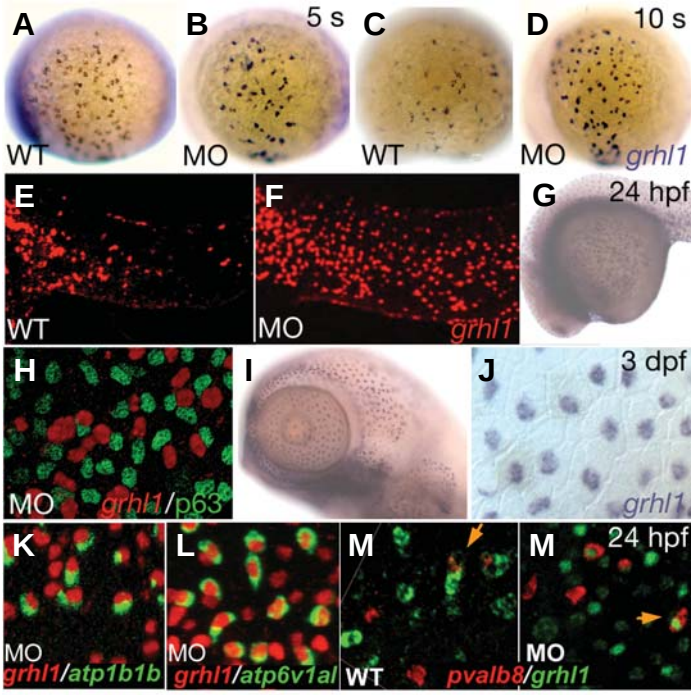
Loss-of-function studies in *Drosophila, Xenopus* and mouse have identified various roles for *grainyhead* and its vertebrate homologues within different developmental processes such as epidermal specification (Bray and Kafatos, 1991; Ting *et al.*, 2003; Tao *et al.*, 2005; Chalmers *et al.*, 2006; Yu *et al.*, 2006), duct maturation of the salivary gland and the kidney (Yamaguchi *et al.*, 2006), as well as position-specific differentiation of *Drosophila* postembryonic neuroblasts (pNBs), influencing timing and duration of proliferation (Almeida and Bray, 2005; Cenci and Gould, 2005; Maurange *et al.*, 2008). In thoracic pNBs *grainyhead* prevents premature cell-cycle exit by repressing *prospero*, a homeodomain protein. Therefore, premature as well as increased expression of *prospero* can be observed in *grh* mutants (Maurange *et al.*, 2008).

To understand the function of *grhl1* in the zebrafish embryo, we analysed the loss-of-function phenotype generated by injection of specific antisense morpholino oligonucleotides (MOs) into the 1-2-cell stage embryo. Two different MOs targeting either the initiation of translation (*grhl1*-ATG-MO) or correct splicing of the gene (*grhl1*-splice-MO) were used. At the concentrations used, the ATG-MO completely blocked translation of co-injected mRNA containing the MO binding site fused upstream of GFP (Supplementary Fig. S1 A-C). The splice-MO blocked correct splicing of the fourth intron, which introduced a premature stop codon before the DNA binding CP2 domain (Supplementary Fig. S1 D-E). In the experiments described below, the ATG and splice MOs were either injected alone or together, and with or without p53 MO to suppress unspecific MO-induced apoptosis (Robu *et al.*, 2007). However, in all conditions, identical results were obtained (Supplementary Fig. S2), suggesting that both morpholinos generated amorphic phenotypes.

The zebrafish homologue of *Drosophila prospero* (see above),

prospero-related-homeobox-gene-1 (*prox1*) is expressed in non-keratinocyte skin cells at 1 dpf (Thisse, 2001; http://zfin.org). However, both in *grhl1* morphants and un-injected controls, its expression started at the 28-somite stage, and no difference in cell numbers or staining intensity was observed at 24 hpf (Supplementary Figs. S3 G,H and S4 D,I). In addition, the spatial and temporal expression patterns of the three aforementioned non-keratinocyte skin cell markers *atp1b1b, atp6v1al* and *pvalb8* appeared unaltered in *grhl1* morphants (Supplementary Fig. S3 A-F and S4 A-C,I). Furthermore, we found normal numbers of *ncx1b*- and *trpv6* (previously called *ecac*)-expressing cells, representing subsets of NaR cells (Pan *et al.*, 2005; Liao *et al.*, 2007; Hwang and Lee, 2007) (Supplementary Fig. S4 E,F,I). Recently, the existence of at least two other skin cell populations, termed NCC (thiazide-sensitive Na^+/Cl^- cotransporter expressing) and GR (glycogen-rich) cells, was proposed (Hwang and Lee, 2007). We have cloned a zebrafish *ncc* gene, termed *ncc22b*, which displays a punctate skin expression from 3 dpf onwards (Supplementary Fig. S4G). However, the number of *ncc22b*-positive cells in *grhl1* morphants was normal (Supplementary Fig. S4G'), as was the expression of *keratin8* (Supplementary Fig. S4H), which is lost in *Xenopus grhl1* morphants (Tao *et al.*, 2005), and which is co-expressed with *grhl1* in the zebrafish periderm (see above; Fig. 1E).

We also tested whether knockdown of *grhl1* affects the function of differentiated ionocytes or the periderm, and thereby the integrity and permeability of the skin, or osmoregulation. Zebrafish larvae with defects during periderm cell differentiation usually display skin shedding (Slanchev *et al.*, 2009). However, the skin of *grhl1* morphants appeared normal during all investigated stages (up to 7 dpf; Supplementary Fig. S5 A,B). Also, osmoregulation seemed normal. While *foxi3a* injected embryos developed oedema around 3 dpf, which could be rescued when larvae were transferred to isotonic Ringers solution (Supplementary Fig. S5 C,D; Hsiao *et al.*, 2007), *grhl1* lacked any signs of oedema formation (Supplementary Fig. S5 A,B). Similarly, when morphants and wild-type animals were exposed to osmotic stress by transfer into 10x embryo medium at 24 hpf, followed by transfer into distilled water at 48 hpf and analysis at 72 hpf, no differences in morphology or survival rates were apparent (Supplementary Fig. S5 E,F). In sum, these results suggest that zebrafish Grhl1 is dispensable for the



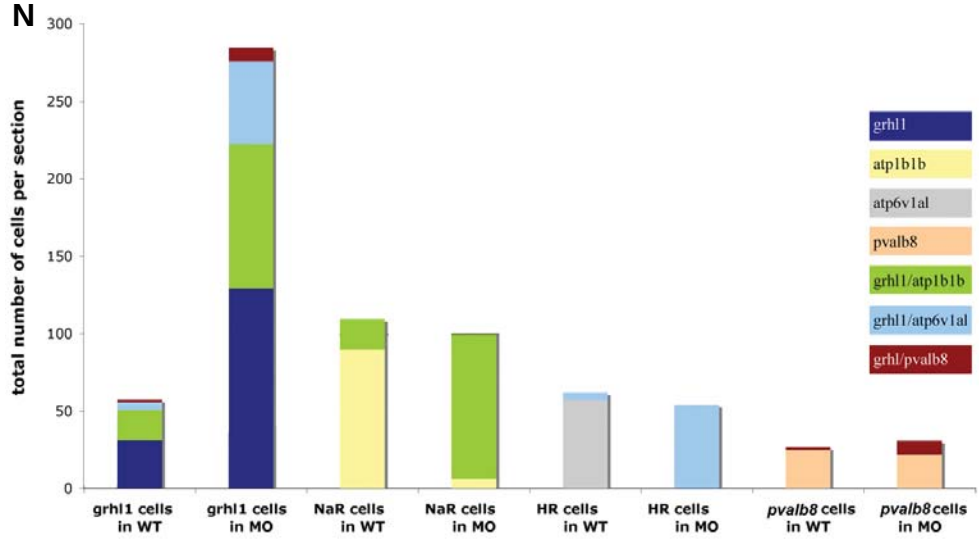


Fig. 6. *grhl1* transcription is controlled in an autoregulatory, time- and cell type-dependent matter. (A-M´) *Lateral views (anterior/ventral to the left) of whole mount* in situ *hybridisations with probes indicated in the lower right corner.* **(H,J,K-M´)** *Cells at the anterior end of the trunk* **(H,K-M´)** *or at the head* **(J)**. **(H)** In situ *hybridisation was followed by an immunostaining for p63. Stages are indicated in the upper right corner and genotypes in the lower left corner and apply to all panels left. MO indicates* grhl1 *morphants. Orange arrows in* **(M,M´)** *indicate cells coexpressing* grhl1 *and* pvalb8. **(N)** *Coexpression of skin cell markers and* grhl1. *It shows a graphical illustration of the average total numbers of* grhl1 *expressing cells (dark blue), NaR cells (yellow), HR cells (grey) and pvalb8 cells (orange), quantified for the left side of the trunk of 10 WT and 10* grhl1 *morphant embryos at 24 hpf. The proportion of co-expressing cells is indicated,* grhl1 *positive NaR cells (green),* grhl1 *positive HR cells (light blue) and* grhl1 *positive pvalb8 cells (dark red). It can be seen that total numbers of ionocytes and pvalb8 cells do not change between WT and morphants, while the proportion of co-expressing cells increases. Please note that in the* grhl1*-splice-morphants shown in this figure, the* grhl1 *mRNA was located primarily in the nuclei. This is in contrast to the cytoplasmic localisation of other mRNAs, see* **(K-M')**, *and in contrast to* grhl1 *mRNA in wild-type (see Fig. 2A) or in* grhl1*-ATG-MO-injected embryos (compare Supplementary Fig. S5C with S5D). Since splicing of hnRNA occurs in the nucleus, incorrectly or not fully spliced* grhl1 *transcripts appear to be incapable of nuclear export. Apart from this different RNA localisation, identical results with progressively increasing numbers of* grhl1*-positive cells were obtained for both* grhl1*-ATG-MO and* grhl1*-splice-MO.*

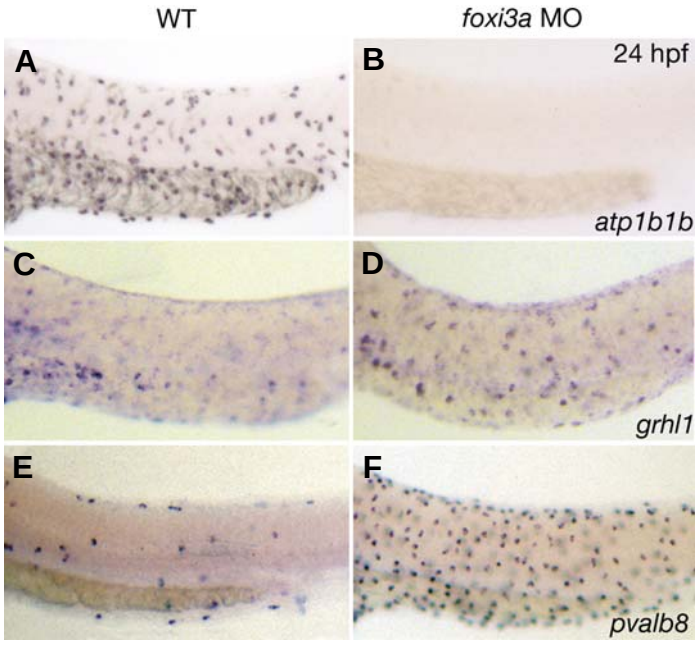


Fig. 7. Loss of *foxi3a* function causes loss of ionocytes and an expansion of *pvalb8*-positive cells, while the number of *grhl1*-positive cells remains unaltered. *All panels show whole mount* in situ *hybridisations at 24 hpf with probes indicated in the lower right corner;* **(A,C,E)** *un-injected controls;* **(B,D,F)** foxi3a *morphants. Anterior is to the left, dorsal up.*

development and function of the different skin cell types.

grhl1 *is required for negative transcriptional autoregulation in differentiating ionocytes*

In contrast to all other investigated skin markers mentioned above, *grhl1* morphants displayed progressively higher numbers of cells positive for *grhl1* itself. At the 5-somite stage, numbers of dispersed *grhl1*-positive epidermal cells in *grhl1* morphants were still normal (Fig. 6 A,B). However, at the 10 somite-stage, cell numbers in morphants were already approximately double as high as those of control embryos (Fig. 6 C and D), while at 24 hpf, the increase was up to five-fold (6E,F and N). In addition to the increase in the number of the dispersed epidermal cells, expression within the periderm cells was stronger and clearly detectable in the entire skin (Fig. 6G). This expression was prolonged until 3 dpf (Fig. 6 I,J). None of the additional *grhl1* cells expressed p63, indicating that they are no keratinocytes from the basal layer (Fig. 6H). In reverse, prominent *grhl1* expression in non-keratinocyte epidermal cells could be largely suppressed upon *grhl1* mRNA injection, while co-expression of *grhl1* mRNA and *grhl1* MO led to a compensation of the two opposite effects, and to wild-type conditions (Supplementary Fig. S6).

The increase in *grhl1* positive single cells in *grhl1* morphants starts around the same time when the co-expression of *foxi3* and *grhl1* begins to decrease in wild-type embryos. This suggest that under wild-type conditions, *grhl1* is down-regulated in ionocyte progenitors by an autoregulatory mechanism during ionocyte differentiation, while this negative feedback is inhibited upon knockdown of the gene. Consistent with this notion, despite the massive increase in the number of *grhl1*-positive cells, the absolute number of BrdU-positive, *grhl1*-positive cells of *grhl1* morphants at 24 hpf remained as in uninjected controls (Fig. 5 D,E), indicating that the supernumerary *grhl1*-positive cells represent differentiated non-keratinocyte skin cells, rather than additional progenitors of the this lineage. Consistent results were obtained via *grhl1* – *ATPase*s double stainings of differentiated ionocytes. While in wild-type embryos, 18 % of the NaR (19.5±3.25 / 109.2±3.76, n=10) and 8 % of HR (5±1.14 / 62.0±2.86, n=10) cells expressed *grhl1* at 24 hpf, this proportion increased in *grhl1* morphants to 94 % (93.25±2.87 / 99.5±1.65, n=10) and 99 % (0.6±0.4 / 54.2±2.26, n=10), respectively (compare Fig. 6K with Fig. 3F; see also Fig. 6 L,N and Supplementary Fig. S7 C-D). However, the overall numbers of both types of ionocytes remained constant, indicating that *grhl1* was up-regulated in these cells rather than additional cells being formed (Fig. 6N). In contrast to ionocytes, the majority of *pvalb8* expressing cells remained *grhl1* negative even in *grhl1* morphants, although an increase of co-expressing cells from roughly 7 % in wild types (1.7±0.4 / 26.7±2.46, n=10) to 28 % in morphants (8.8±2.92 / 30.8±2.65, n=10) could be detected (Fig. 6 M,M´,N). The presence of *grhl1*-negative *pvalb8* cells suggests that in this cell-type, and in contrast to ionocytes, *grhl1* expression can be switched off via another mechanism that acts in parallel to the described negative Grhl1 autoregulation.

foxi3a *morphants display normal numbers of* grhl1*-, but significantly increased numbers of* pvalb8*-positive cells*

The data described thus far indicate the presence of different non-keratinocyte skin cell lineages, all of which derive from the same pool of epidermal precursors, and all of which display transient expression of *grhl1*. In contrast, expression of *foxi3a* and *foxi3b* is restricted to a subset of this population, driving the specification of cells towards the NaR and HR ionocyte fate. Knockdown of *foxi3* leads to a loss of ionocytes, indicated by the absence of *atp1b1b* and *atp6v1a1* expression at 24 hpf (Fig. 7 A,B; Hsiao *et al.*, 2007; Jänicke *et al.*, 2007). In contrast, knockdown of *foxi3a* or *foxi3b* or *both* did not affect the numbers of *grhl1* positive cells at 24 hpf (Fig. 7 C,D and data not shown). Most strikingly, *pvalb8*-expressing cells even displayed a positive response, opposite to that of NaR and HR ionocytes, with significantly increased numbers in *foxi3a* morphant embryos at 24 hpf (Fig. 7 E, F). These findings are in line with the notion that *grhl1* is a common marker for both Foxi3-promoted (NaR and HR) and Foxi3-suppressed (*pvalb8*-positive) cells, and that Foxi3a may act as a molecular switch converting cells that would otherwise express *pvalb8* to NaR or HR ionocytes.

Discussion

The Grh/CP2 family of transcription factors is widely conserved within the animal kingdom and has been implicated in many developmental pathways such as terminal differentiation of the skin and neurogenesis (Bray and Kafatos, 1991; Cenci and Gould, 2005; Tao *et al.*, 2005; Maurange *et al.*, 2008). In this study, we have analysed the conservation of the family in the zebrafish genome and investigated the expression and function of *z-grhl1* in the zebrafish skin, with special focus on non-keratinocyte cell lineages and the relationship between

Grhl1 and the known ionocyte regulator Foxi3. To our knowledge, no Grh/CP2 member has been described in this context in any organism before.

Seven members of the Grh/CP2 family exist within the zebrafish genome

During early vertebrate evolution, two rounds of whole-genome duplication are thought to have occurred, generating four homologues of each invertebrate gene, some of which were subsequently lost (reviewed in Meyer and Schartl, 1999). Consistent with this model the Grh/CP2 family has expanded from one gene, *Ce-grh1*, in *C. elegans*, and two members, *D-grh* and *D-Cp2*, in *Drosophila,* to six homologues, named *Grhl1-3*, *Cp2*, *Cp2-like1* and *Lbp-1a* in mammals (Bray and Kafatos, 1991; Wilanowski *et al.*, 2002; Venkatesan *et al.*, 2003).

In the teleost branch, a third round of whole-genome duplication took place, although many of duplicated genes were not maintained during further evolution (Postlethwait *et al.*, 1998). We have identified seven homologues of *D-Grh/Cp2* within the zebrafish genome (Fig. 1). Two of those, *grhl1* and *z-cp2,* had been previously proposed based on partial EST sequences (Venkatesan *et al.*, 2003). Upon alignment with published invertebrate and vertebrate *Grhl/Cp2* genes, the seven zebrafish genes clustered with the six known mammalian groups described above (Fig. 1). The fact that no seventh or eighth group was identified confirms that the radiation of the invertebrate *grainyhead* and *Cp2* gene had already occurred in the ancient vertebrate lineage prior to the split of the actinopterygia, ray-fined-fish, and the sarcopterygian, which include tetrapodes and lungfish. Our analysis further suggests that most paralogues of the *grhl/CP2* genes generated during the third round of genome duplication were not maintained in the zebrafish lineage, as only one duplicated pair was found, *grhl2a* and *grhl2b*. These two paralogue genes show different expression patterns, which indicates that they may have adopted different functions and therefore were both subject to selective pressure and hence were retained. While *grhl2b* is ubiquitously expressed, expression of *grhl2a* is restricted to the olfactory and otic placodes as well as to the developing pronephros (Fig. 1). The mouse homologue, *grhl2*, is also expressed in the developing kidney (Auden *et al.*, 2006; Yamaguchi *et al.*, 2006) indicating a potential conserved role of *grhl2* during kidney development.

Zebrafish* grhl1 *and* grhl3 *are transiently expressed in the periderm

In *Xenopus* and mouse, members of the Grhl subfamily are involved in skin development. In *Xenopus*, expression of *Grhl1* and *Grhl3* is found in the non-neural ectoderm and later in the superficial layer of the epidermis (Tao *et al.*, 2005; Chalmers *et al.*, 2006), which was proposed to be a homologous structure to the zebrafish periderm (Sagerstrom *et al.*, 2005). Within the skin of mice *Grhl1* and *Grhl3* are expressed in all suprabasal layers, which are formed by terminally differentiating cells deriving from the basal cells, and detected from day E8.5 with progressively increasing expression. In contrast, *Grhl2* expression also starts at E8.5 but decreases until E12.5, and is restricted to the basal layer, the stratum spinousum and the stratum granulosum (Auden *et al.*, 2003).

Of the two epithelial layers of the zebrafish epidermis, *grhl1* is only expressed in the outer periderm, but not in the basal layer (Fig. 1), similar to the situation in *Xenopus*, and similar to the basal exclusion of *Grhl1* in mouse. Furthermore, the zebrafish periderm displays at least temporary co-expression of *grhl1* with *grhl3*, although the temporal expression profiles of the two genes appear quite different, with an earlier onset and cessation of *grhl3* expression. This temporal co-expression of the two paralogs in the zebrafish periderm again is very similar to the behaviour of their orthologs in the outer skin layer in *Xenopus* (Tao *et al.*, 2005; Chalmers *et al.*, 2006) and in the suprabasal layers in mouse (Auden *et al.*, 2003).

grhl1 ***is transiently expressed in specifying ionocytes***

In addition to the periderm, zebrafish *grhl1* is highly expressed in single cells spread across the basal epidermis in a punctate pattern, starting at early segmentation stages (Fig. 1). Several lines of evidence suggest that some of these cells are precursors of NaR and HR-type ionocytes. We and others have previously shown that precursors of these ionocyte types express *foxi3a* and *foxi3b*, which encode transcription factors required for ionocyte differentiation and possibly *ATPase* gene activation (Jänicke *et al.*, 2007; Hsiao *et al.*, 2007). During early segmentation stages (up to the 10-somite stage), all *foxi3*-positive cells co-express *grhl1* (but not all *grhl1*-positive cells co-express *foxi3a*; see below). However, during further development, more and more *foxi3*-positive, but *grhl1*-negative cells show up, suggesting that *grhl1* is switched off during early steps of ionocyte differentiation (Fig. 3). Accordingly, wild-type larvae display only a few cells with *grhl1*/*atp1b1b* or *grhl1*/*atp6v1al* coexpression (18 % of NaR cells and 8 % of HR; Figs. 3 and 6). The co-existence of *foxi3*/*grhl1* and *ATPase*/*grhl1* double-positive cells together with *foxi3*- and *ATPase* -positive cells lacking *grhl1* transcripts provides further support to our previously proposed model that the neo-formation of ionocytes is a continuous process (Jänicke *et al.*, 2007). Consistently, we have shown here that *grhl1*-positive cells can proliferate and incorporate BrdU even during mid segmentation stages, in co-existence with post-mitotic, fully differentiated non-keratinocyte cell types that are all BrdU-negative (Fig. 5). Important further evidence for a transient *grhl1* expression in <u>all</u> HR and NaR ionocytes comes from our analysis of *grhl1* morphants (Fig. 6). Although other, more complicated interpretations cannot be completely ruled out, these data strongly suggest that Grhl1 protein is required for the down-regulation of *grhl1* transcription during ionocyte differentiation. Upon knock-down of *grhl1, grhl1* transcription is maintained and can be used as a lineage tracer, now marking almost all NaR and HR type ionocytes even during later stages of development (compare Fig. 3 with Fig. 6, and see Fig. 6N for quantification). To our knowledge, this is the first report of a negative autoregulative mechanism for any grh/CP2 family member and in any organism. Such regulatory feedback loops seem a very common regulative mechanism during vertebrate development, and been shown for multiple other zebrafish transcription factors such as Hoxb1a, Pax6 or Gata1 (McClintock *et al.*, 2002; Aota *et al.*, 2003; Kobayashi *et al.*, 2001). Future studies have to show whether the negative Grhl1 autoregulation is direct or mediated by other transcription factors. Interestingly, sequence analysis of the *grhl1* gene revealed a 33 bp DNA element approximately 5 kb upstream of the transcriptional start site with 60.6% identity (20/33) with a previously described essential Grhl1 binding site in the *Xenopus* keratin gene XK81A1 (Huang *et al.*, 1995; Tao *et al.*, 2005) (Supplementary Fig. S8 A-C). This upstream region, as well as the putative

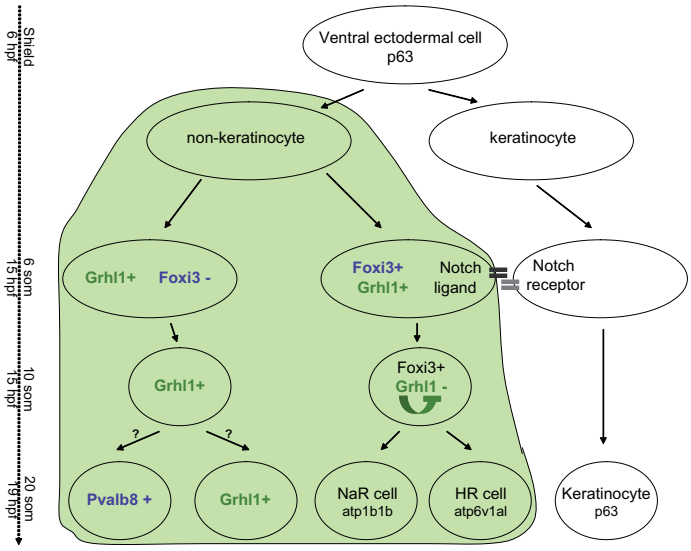


Fig. 8. Model summarising known and proposed mechanisms driving the sequential segregation of different epidermal cells types of the zebrafish embryo. *This model is based on a combination of previously described data (Hsiao* et al.*, 2007; Jänicke* et al.*, 2007), and data presented here (highlighted in bold). The developmental time points are indicated on the left. All non-keratinocyte epidermal cells labelled by* grhl1 *are on a light green background. The mechanisms driving their segregation from the common pool of ventral ectodermal cells are not known, but might involve stochastic events. The Grhl1 lineage further segregates into a foxi3-positive and a foxi3a-negative sub-lineage. The mechanisms restricting the initiation of* foxi3 *expression to a subset of the* grhl1 *population remain unclear, however, the involvement of Notch signalling can be ruled out (see text for further discussion). Whether the* foxi3-*negative* grhl1 *cells exclusively differentiate into* pvalb8 *cells or additional, thus far unknown epidermal cell types, remains unclear and is indicated by question marks (see text for further discussion). According to previous work, the Foxi3 lineage employs lateral inhibition via Notch signalling to prevent a further expansion of NaR/HR ionocytes at the expense of keratinocytes (Hsiao* et al.*, 2007; Jänicke* et al.*, 2007). This does not seem to be the case for* pvalb8 *cells, which according to our unpublished data do not respond to interferences with Notch signalling. The Foxi3 lineage, but not the non-Foxi3 lineage, further displays negative auto-regulation of* grhl1 *expression. Whether this negative autoregulative autoregulation is required for NaR/HR ionocyte differentiation is unknown.*

promoter region, also contains multiple potential binding sites for Foxi1 (T(g/a)TTT(g/a)(t/c); Kurth *et al.*, 2006), a close Foxi3 relative (Supplementary Fig. S8 A,D). Recently, zebrafish *foxi1* has been shown to be required upstream of the *foxi3* genes for skin ionocyte development (Esaki *et al.*, 2009). It will be interesting to elucidate the functional connection between *foxi1* and *grhl1*. If Foxi3 proteins have a direct effect on *grhl1* transcription in ionocytes, it most likely is positive, rather than negative, as suggested by our unpublished observation according to which the punctate *grhl1* expression is absent, rather than more persistent, in *foxi3* morphants.

grhl1 *is transiently expressed in other non-keratinocyte epidermal cell lineages*

In *grhl1* morphant embryos with prolonged *grhl1* expression, only approximately half of the *grhl1* population was ATPase-positive and thus belonged to the ionocyte lineage. The exact nature and composition of the other half is not totally clear. Some of the cells might represent immature ionocytes (see above), while others belong to the *pvalb8*-lineage, indicated by *grhl1*/*pvalb8* co-expression at 24 hpf (Figs. 4,8). *pvalb8* has been previously described as a marker of epidermal mucous cells, an assumption that was solely based on its punctate epidermal expression pattern (Hsiao *et al.*, 2002). Also, Pvalb8 itself does not allow definitive conclusions about the nature of the cells. It represents a Ca^{2+} binding protein that is thought to be a cytosolic ion reservoir modulating Ca^{2+}-dependent signalling pathways (reviewed by Pauls *et al.*, 1996; Zacchia and Capasso, 2008). However, our double *in situ* analyses show that *pvalb8*, *atp1b1b* and *atp6v1al* are indeed expressed in a mutually exclusive manner, identifying the *pvalb8* cells at least as a distinct epidermal population. Furthermore, according to our lineage tracing experiments, they derive from the same population of epidermal precursor cells like keratinocytes and ionocytes (Fig. 4). In this light, *grhl1* appears as a more general marker of non-keratinocyte cell types, in contrast to *foxi3a* and *foxi3b*, which are restricted to NaR and HR ionocytes (Fig. 8). Future experiments have to reveal whether *grhl1* is also expressed in progenitors of further, thus far uncharacterized epidermal cell lineages. Such a lineage has been recently proposed by the Hwang laboratory and named NCC cells, while nothing is known about its embryonic origin, and no markers are available to stain cells prior to 3 dpf (reviewed in Hwang and Lee, 2007; see Supplementary Fig. S4 of this study).

Foxi3 plays a crucial role in subdividing the Grhl1 population by promoting NaR/HR ionocyte specification while blocking the pvalb8 *fate*

We and others have previously shown that Foxi3 is required for NaR/HR ionocyte differentiation (Hsiao *et al.*, 2007; Jänicke *et al.*, 2007). Here, we show that the loss of ionocytes in *foxi3a* morphants is accompanied by a gain of *pvalb*8 cells, while the number of cells expressing the common marker *grhl1* remains unaltered (Fig. 7). This suggests that *foxi3* not only promotes the ionocyte fate, but also suppresses the *pvalb8* fate, meaning that Foxi3 induces a switch from the "default" *pvalb8* to the NaR/HR ionocyte fate (Fig. 8). Similar fate-switching effects have been described for multiple other transcription factors, such as the Pou-homeodomain protein Pit1, which converts adenohypophyseal cells from their "default" gonadotrope or corticotrope to the somatotrope, lactotrope or thyrotrope fate by activation and repression of the corresponding hormone gene (Nica *et al.*, 2004; and references therein).

Currently, we can only speculate about the mechanisms regulating the differential expression of *foxi3* genes in some, but not all of the *grhl1* cells. However, it is interesting to note that at 24 hpf the ratio of *atp1b1b/grhl1* and *atp6v1al/grhl1* double positive versus *ATPase*-negative *grhl1* cells is close to 50:50 (Fig. 6N), and that in our lineage tracing experiments, epidermal clones often contained two different non-keratinocyte (p63-negative) cell types next to each other, a *pvalb8*-positive and a *atp1b1b* or *atp6v1al*-positive cell (Fig. 4 D-F). Thus, it is tempting to speculate that *foxi3*-positive and *foxi3*-negative *grhl1* cells might have the same mother cells (Fig. 8). Consistent with this notion, we found that some of the *grhl1*-positive cells incorporated BrdU during mid-segmentation stages (Fig. 5), thus, after they might have been singled out from the pool of epidermal progenitors by the Notch system (Jänicke and Hammerschmidt, 2007). It is tempting

to speculate that these rather late cell divisions might be the ones that give rise to a ionocyte and a *pvalb8* cell from a singled-out non-kerationcyte precursor. During development, such binary cell fate determination processes are rather common mechanisms, most of which involve a combination of asymmetric localization of intrinsic factors in the mother cells, such as the Notch signalling modulators Numb and Neutralized or the aforementioned Prospero (Le Borgne and Schweisguth, 2003; Manning and Doe, 1999), and differential extrinsic signalling on sibling cells, e.g. via Notch or Epithelial Growth Factor (EGF) (Berset *et al.*, 2005; Shin *et al.*, 2007). We found normal numbers of *pvalb8*-positive cells in mutants deficient for Notch signalling and after constitutive Notch activation (unpublished own data; compare with Jänicke *et al.*, 2007), suggesting that such a binary pvalb8-NaR/HR cell fate decision within the *grhl1* population of non-keratinocyte epidermal cells is independent of the Notch system. More studies will be necessary to unravel the exact mechanisms underlying these sub-functionalisation events.

grhl1 *morphants do not show discernable defects*

Similar to the temporal expression profile described here for zebrafish *grhl1*, *Drosophila grh* displays transient expression in specific progenitor cells, e.g. in postembryonic neuroblasts (pNBs), while expression ceases when the cells stop to divide (Uv *et al.*, 1997). Mutant analyses further revealed that *grh* is required for production of the correct number of pNBs, dependent on their temporal and spatial position within the larvae. While the lineage of thoraic pNBs decreased in mutants, the numbers of abdominal pNBs increased (Almeida and Bray, 2005; Cenci and Gould, 2005; Maurange *et al.*, 2008). It is tempting to speculate that in zebrafish *grhl1* functions in a similar way to its *Drosophila* homologue and is involved in correct timing of differentiation of non-keratinocyte skin cell lineages.

However, knock-down of *grhl1* via injection of different antisense MOs did not yield any discernable defects in the timing of differentiation or the final numbers of the different non-keratinocyte epidermal cells (Supplementary Figs. S3-S5). Furthermore, in contrast to *Xenopus* and mouse *Grhl1* (Tao *et al.*, 2005; Wilanowski *et al.*, 2008), we found zebrafish *grhl1* to be dispensable for keratinocyte development.

We can only speculate about the reasons for the dispensable nature of zebrafish *grhl1*. It is possible that another member of the family or a different transcription factor can compensate for loss of *grhl1*. Knockout of mouse CP2 does not lead to any obvious phenotype, and it was shown that the highly similar paralogue LBP-1a can compensate for loss of CP2 in mouse and human cell culture (Ramamurthy *et al.*, 2001; Kang *et al.*, 2005). A candidate accounting for such functional redundancy in ionocytes and *pvalb8* cells of the zebrafish could be *grhl2b*, which is ubiquitously expressed during the first days of development (Fig. 1). In the periderm, there could be functional redundancy between *grhl1* and *grhl3*, which are coexpressed in this outer layer of the embryonic skin, but not in ionocyte and *pvalb8* cell precursors (Fig. 1; see also above). *grhl1* and *grhl3* double knock-down experiments are in progress to test this possibility. In mouse, both Grhl1 and Grhl3 are essential for skin development (Tao *et al.*, 2005; Ting *et al.*, 2005; Hislop *et al.*, 2006; Yu *et al.*, 2006; Yu *et al.*, 2008). This suggests that during vertebrate evolution, the two paralogs might have given up their initially redundant roles to fulfil specialised functions in the more complex mammalian skin.

Whether the observed indispensable role of Grhl1 as a negative auto-regulator of its own expression in non-keratinocyte epidermal cells is of biological relevance is unclear, since in our morphant animals, the persistently generated *grhl1* transcripts are inactive. To look into this, it would be necessary to force prolonged *grhl1* expression in differentiating epidermal cells. One attractive possibility consistent with some of the fly data would be that such prolonged expression leads to delayed ionocyte differentiation and/or further progenitor proliferation. Our preliminary experiments with *grhl1* mRNA injection did not have any effects apart from a reduction of endogenous *grhl1* transcription (Supplementary Fig. S6; and unpublished observations). However, this might be due to insufficient stability of the injected RNA (up to early segmentation stages; Harland and Misher, 1988; Fink *et al.*, 2006), and does not necessarily mean that the observed down-regulation of *grhl1* expression in differentiating ionocytes is dispensable.

Materials and Methods

RT-PCR

foxi3a, foxi3b, grhl1, grhl2a, grhl2b, grhl3, cp2, cp2-like1, lbp-1a, prox1, ncx1b, and *ncc22b* cDNA fragments were amplified via RT-PCR with total RNA isolated from zebrafish embryos at 12, 24, 48 and / or 72 hpf, and the primers listed in Supplementary Table 1.

Labelling procedures

Whole mount *in situ* hybridisations and immunostainings were performed as previously described (Hammerschmidt *et al.*, 1996). *In situ* hybridizations for each *grhl* gene were carried out with probes of two different lengths (short: 300-600 bp; long: 600 – 1000 bp) and at two different stringencies (moderate: hybridization in 5xSSC, 50% formamide at 55°C, wash in 0.2xSSC at 55°C; high: hybridization in 5xSSC, 60% formamide at 70°C, wash in 0.1xSSC at 72°C). Under all conditions, identical results were obtained. DNp63 protein was detected with the mouse anti-p63 antibody 4A4 (Santa Cruz) as previously described (Bakkers *et al.*, 2005). For *in situ* hybridisations the following riboprobes were used: *col1a1* (Fisher *et al.*, 2003; Jänicke *et al.,* 2007), *atp1b1b* and *atp6v1al* (Jänicke *et al.,* 2007), *trpv6* and *pvalb8* (clone IRAKp961C14226Q and IRBOp991F0754D, ImaGene, Germany; probe synthesis by PCR following the protocol on ZFIN.org). For the *foxi3a, foxi3b, grhl1, grhl2a, grhl2b, grhl3, cp2, cp2-like1, lbp-1a, prox1, NCX1b, NCC22b* riboprobes, RT-PCR fragments (see above) were ligated into pGEMTeasy (Promega), and plasmids were linearised and transcribed with enzymes listed in Supplementary Table 1. For single *in situ* hybridisations, probes were labelled with digoxygenin (Roche). In addition, for double *in situ* hybridisations, riboprobes for *foxi3a, foxi3b, grhl1, atp1b1b* and *atp6v1al* were labelled with fluorescein (Roche).

Fluorescent double *in situ* hybridisations were performed as described previously (Jänicke *et al.*, 2007). Photographs were taken with a Zeiss LSM510 META confocal microscope.

Bromodeoxyuridine (BrdU) labelling

Embryos were dechorionated on petri dishes coated with 1 % agarose and incubated in 2 mg/ml BrdU + 2 % DMSO in embryo medium from 16 hpf (15-somites stage) to 24 hpf (30-somites stage) at 28°C. At 24 hpf embryos were briefly washed in embryo medium and fixed in 4% PFA at 4°C over night. For detection, *in situ* hybridisation was performed as before. Digoxygenin-labelled probes were developed with FastRed (Sigma). Once the desired staining intensity was achieved, embryos were washed twice for 5 min in PBST and incubated in 2N HCl for 1h at 37°C. After 5 washes with PBST for 5 min each, embryos were blocked in PBST

supplemented with 2% sheep serum/ and 2mg/ml BSA for 1h at RT, and incubated in anti-BrdU-Alexa488 antibody (Molecular Probes) at a 1:100 dilution at 4°C over night. Stainings were analysed and photographs taken with a Zeiss LSM510 META confocal microscope.

Morpholino and synthetic mRNA injections

For loss-of-function analysis antisense morpholinos (MO) were purchased from GeneTools and injected into 1-2 cell stages as described previously (Nasevicius and Ekker, 2000). Sequences of *foxi3*-MOs and *p53*-MO were described previously (Jänicke *et al.*, 2007; Plaster *et al.*, 2006). Two *grhl1*-MOs were used in this work, targeting the splice donor of intron 3-4 or the ATG start codon, respectively. The sequences were: CTT TGA TGA GAG CTT CAC CTT TTG T (*grhl1*-splice-MO) and GTG ACA TCT CTT ATG GTC GAA CTG G (*grhl1*-ATG-MO). Unless stated otherwise, the ATG-MO was used. Per embryo 3 ng of MO were injected (*foxi3a*, *foxi3b*, *grhl1*-splice, *grhl1*-ATG).

For *grhl1* overexpression, the coding region of *grhl1* was amplified via RT-PCR with the primers listed in Supplementary Table 1, cloned into the plasmid pCS2+ (grhl1-FL). To validate the specificity and efficacy of the *grhl1*-ATG-MO, the MO binding site and its flanking up- and downstream regions were amplified from cDNA with primers listed in Supplementary Table 1. The sequence was cloned into the XLT.GFP.CS2+ vector (generous gift of Jeff Miller), so that the ATG translation initiation codon of the amplified sequence was in frame and upstream of the GFP coding sequence (grhl1-GFP). pCS2-based plasmids were linearised with *EcoR*I and capped RNA was synthesised using the T3 message machine kit (Ambion, USA). Synthetic mRNA was injected into the 1-cell stage either alone or combined with the specific ATG-MO, targeting the cloned sequence, or a control ATG-MO, targeting a different sequence. Embryos were screened for GFP at late blastula stages using a dissecting microscope. At the MO concentrations used in this work, the MO efficiently inhibited translation of GFP from the specific construct, while translation was not inhibited upon injection with an unspecific MO.

To control for the efficacy of the *grhl1*-splice MO, RT-PCR was performed on RNA extracted from wild-type or morphant embryos with primers binding in exon3, grhl1-ex3-for (GAC AAG AGA ACA ATA TCC CAG C) and in intron3-4, grhl1-int3/4-rev (TCA AAA ATC CTA TCA GTA AAT TT).

Lineage tracing experiments

Lineage tracing experiments were performed as previously described (Jänicke *et al.*, 2007), transplanting single cells from *bactin:mGFP* transgenic donor embryos (Cooper *et al.*, 2005) into non-transgenic hosts. To obtain single cells, up to 10 cells were pulled from the donor embryo into the transfer needle and separated from each other by multiple up- and down movements within the pipette. Afterwards, all but one cell were released into the medium under optical control, and the remaining single cell was transferred into the host embryo. Recipients were fixed at 24 hpf, followed by fluorescent *in situ* hybridisation for *pvalb8* as described above, by fluorescent immunolabelling of keratinocytes (primary mouse-anti-p63 antibody 4A4 (Santa Cruz) and anti-mouse-Alexa647; Molecular Probes), and by fluorescent immunolabelling of the descendants of transplanted cells (primary rabbit anti-GFP antibody and secondary anti-rabbit-Alexa488 antibody, Molecular Probes).

Osmoregulation experiments

For morphology analysis, fish were injected with MOs as described above and raised in 1x Embryo medium until the day of analysis. For osmotic stress experiments, fish were injected and raised in 1x Embryo medium until 24 hpf. They were removed from their chorions and transferred into 10x Embryo Medium for 24 hpf. At 48 hpf, the medium was replaced by distilled water. Morphology and survival rate were assessed at 72 hpf.

Quantification of gene expression

For quantification of marker expression all cells covering both sides of the trunk and the yolk sac extension of WT and *grhl1* morphant embryos at 24 hpf and 72 hpf were counted for 5 embryos each time. For quantification of co-expression all cells covering the anterior part of the trunk and the entire yolk sac extension of WT and *grhl1* morphant embryos at 24 hpf were counted for 10 embryos each time. Analysis was performed using Excel software; statistical significance was determined using the Student's *t* test.

Database searches

The zebrafish assembly version 7 (Zv7) was searched by BlastN and TBlastN (Altschul *et al.*, 1997), using *D-grh, D-Cp2* and the six human genes as a query. The GenBank accession numbers of the zebrafish grhl/cp2 genes identified in this study as well as previously published homologues are listed in Supp-Table 2. For identification of zebrafish *ncc* genes, searches were performed using the human *SLC12A3* (NM_000339) as a query. This identified a putative *ncc* gene on chromosome 18 (NM_001045080). When this sequence was used in an additional search, 3 more putative *nccs* were identified on chromosome 22, termed here *ncc22a* (XM686141), *ncc22b* (NM001045001) and *ncc22c* (XM001342852). Primers were designed for all four candidates, but only *ncc22b* could be amplified from 2 dpf cDNA.

Phylogeny analysis

All sequence analyses were performed using programs from the PHYLIP package (version 3.5c) (Felsenstein, 1989) available on the Mobyle portal of the Pasteur Institute, Paris (http://bioweb2.pasteur.fr/intro-en.html). Protein sequences were aligned using ClustalW (Thompson *et al.*, 1994). For construction of trees, regions that could not be aligned across all sequences were discarded from the alignment. For calculation of the trees, a discrete data method, parsimony, and a distance-matrix method, neighbor-joining, were compared; neighbor-joining created trees with highest bootstrap values. Statistical significance was assessed by 500 bootstrap replications (Thompson *et al.*, 1994) and percent values were included at the major branching points in the tree.

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